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⑰ Applicant: **Degussa Aktiengesellschaft**  
**Weissfrauenstrasse 9**  
**D-6000 Frankfurt am Main 1(DE)**

⑱ Inventor: **Bachmann, Bernd, Dr.**  
**Meyerfeld 10a**  
**D-4806 Werther(DE)**  
Inventor: **Thierbach, Georg, Dr.**  
**Gunststrasse 21**  
**D-4800 Bielefeld 1(DE)**  
Inventor: **Kalinowski, Jörn**

**Drögestrasse 25**

**D-4800 Bielefeld(DE)**

Inventor: **Pühler, Alfred, Prof. Dr.**

**Am Waldschlösschen 2**

**D-4800 Bielefeld 15(DE)**

Inventor: **O'Reagan, Mike**

**3 rue de Schaffhouse**

**F-67000 Strasbourg(FR)**

Inventor: **Viret, Jean Francois**

**26 rue des Muguets**

**F-67380 Lingolsheim(FR)**

Inventor: **Lepage, Pierre**

**1 rue de la Division Leclerc**

**F-67000 Strasbourg(FR)**

Inventor: **Lemoine, Yves**

**4 rue des Alisiers**

**F-67000 Strasbourg-Neudorf(FR)**

② DNA fragment coding for phosphoenolpyruvate carboxylase, recombinant DNA carrying said fragment, strains carrying the recombinant DNA and method for producing L-amino acids using said strains.

③ The invention relates to a DNA fragment coding for phosphoenolpyruvate carboxylase, recombinant DNA carrying said fragment, strains of the genus *Corynebacterium* or *Brevibacterium* carrying the recombinant DNA and method for producing L-amino acids using said strains.

**EP 0 358 940 A1**

1a.

## DNA FRAGMENT

## Background of Invention

## Field of Invention

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The present invention relates to a DNA fragment isolated from a *C. glutamicum* strain coding for PEPC and to recombinant DNA carrying said fragment, strains carrying the recombinant DNA and to the method for producing L-amino acids using said strains.

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## Description of the Prior Art:

The enzyme phosphoenolpyruvate carboxylase (EC 4.1.1.3.1 ; PEPC) is of particular interest in the metabolism of amino acids, since it is involved in a so-called anaplerotic function, which ensures a constant supply of oxaloacetate to the cell.

Oxaloacetate in turn occupies a central position in the metabolism of amino acids, both as the immediate precursor of L-aspartate and as a member of the tricarboxylic acid (TCA) cycle. Indeed, amino acids such as L-lysine, L-methionine, L-threonine and L-isoleucine derive from L-aspartate in a series of branched and highly interregulated biosynthetic pathways, whereas amino acids such as L-glutamate, L-glutamine, L-proline, L-arginine, L-citrulline, L-ornithine etc. derive from intermediates of the TCA cycle.

Thus, PEPC activity is implicated in the biosynthesis of all the amino acids hereabove mentioned.

It is apparent from the above considerations that the biosynthetic levels of amino acids, such as L-lysine, might vary depending on the intra-cytoplasmic specific activities of enzymes such as PEPC.

Considering the important role played by PEPC in the biosynthesis of amino acids, it has always been desirable to try to provide improved means for the production of amino acids by increasing the activity of PEPC.

For example, European Patent Application EP-A-0143195 discloses the cloning of the *ppc* gene isolated from *Brevibacterium lactofermentum* ATCC 13869 and the transformation of bacteria of the genera *Corynebacterium* with a recombinant plasmid, carrying said gene in order to produce L-lysine or threonine.

The prior art has also described *Corynebacterium melassecola* strains transformed with a recombinant plasmid DNA carrying the *ppc* gene of *C. melassecola*.

These strains show an increased PEPC activity, but no evidence is provided for an increase of amino acid production (FR-A-2581653).

There is no suggestion in these publications to enhance the fermentative production of amino acids, especially L-lysine, by cloning the *ppc* gene isolated from *Corynebacterium glutamicum*.

## Summary of the Invention:

The inventors have discovered that, when introducing the genetic information coding for PEPC into an appropriate vehicle capable of replicating in *Corynebacteria* or *Brevibacteria*, and the resulting hybrid vehicle carrying said genetic information is replicated in an appropriate *Corynebacterium* or *Brevibacterium* host or recipient, the transformed microorganism is an excellent producer of L-amino acids, especially L-lysine.

This invention is of particular interest since many strains of *Brevibacterium* and *Corynebacterium* genera producing high amounts of L-amino acids can be utilized as hosts.

The invention thus relates to a process for the fermentative production of L-amino acids and to the various genetic and microbiological elements involved in said process. For example, the invention relates to the isolated form of the gene for PEPC, to various vehicles containing said gene, which vehicles are replicable in above mentioned bacteria, to various microcosms of said microorganisms containing such vehicles, and to various fermentation processes for the production of L-amino acids.

## Description of the Preferred Embodiments:

The present invention relates to a DNA fragment isolated from a *C.glutamicum* strain containing a genetic sequence comprising information coding for the production of a protein having the activity of phosphoenolpyruvate carboxylase (PEPC).

The donor may be one which is either mutated in *ppc* gene, or which is wild type in *ppc* gene.

The DNA fragment consists essentially of 3422 base pairs, is flanked at its termini by *Sal* I restriction sites and is coding for the production of PEPC, which shows the N-terminal amino acid sequence Thr<sup>1</sup> - Asp - Phe - Leu - Arg<sup>5</sup> - Asp - Asp - Ile - - Arg - Phe<sup>10</sup> - Leu - Gly - Gln - Ile - Leu<sup>15</sup>.

The structural gene coding for PEPC consists of 2757 base pairs.

The PEPC of interest is not stimulated by acetyl CoA.

A recombinant DNA containing a DNA fragment consisting of the *ppc* gene can be constructed according to conventional methods for example, by digesting chromosomal DNA and vector plasmid with a restriction enzyme, followed by treatment with a DNA ligase or by digesting chromosomal DNA and vector plasmid with a restriction enzyme, followed by treatment of cleaved terminals with terminal transferase, DNA polymerase, etc., and successive treatment with a DNA ligase, etc. (Methods in Enzymology 68 (1979)).

To isolate the *ppc* gene, a genomic bank of *C.glutamicum* ATCC 13032 was constructed in the plasmid pUC18, a vector commonly used for cloning in the gram negative bacterium *E. coli*. The *ppc* gene was isolated by complementation of known *E. coli* mutant strains affected in the corresponding genes. Candidates clones were analysed and shown both genetically and enzymatically to bear inserts containing the sought after gene.

The *ppc* gene was later subcloned onto so-called plasmid shuttle vectors (vehicles) allowing its propagation in both *E. coli* and the original *Corynebacterium* host, or any type of glutamate producing strain, in order to provide a recombinant DNA molecule containing the new DNA fragment inserted in a vector capable of replication in a glutamate producing strain.

Of particular interest are the vectors pZ1 and pCV 22.

Plasmid pZ1 comprises a drive unit region capable of propagating in *Coryneform* glutamic acid producing bacteria and *E.coli*, and having at least a region to express resistance to a drug.

pZ1 is disclosed in the German Patent Application 37 37 729.9.

pCV 22 is an essentially pure plasmid, which is characterized by a length of 4,5 kb and a restriction endonuclease cleavage chart shown in Figure 6.

The vectors can be obtained from the cells of microorganisms on deposit, by lysing the cells according to the state of art.

The recombinant DNA containing the *ppc* gene of wild type or mutant type can be introduced into microorganisms preferred of the genus *Corynebacterium* or *Brevibacterium* by known transformation methods.

Vehicles capable of replication in said microorganisms are the plasmids pDM 2 or pDM 6 (restriction maps shown in Figure 5 and 7).

*C.glutamicum*, *C.melassecola*, *B.lactofermentum* and *B.flavum* are preferred as recipients or hosts, especially bacteria already known for the production of amino acids.

For the expression of the *ppc* gene in the transformed strains, any promoter known as efficient in *Corynebacterium* or *Brevibacterium* can be used. They may be endogenous promoters of these strains, that is promoters controlling the expression of genes originally belonging to the strain. They may also be exogenous promoters, among which the promoter *ptac*, *plac*, *ptrp*, *P<sub>R</sub>* and *P<sub>L</sub>* of phage  $\lambda$ , can be mentioned.

A further object of the invention is a process for the production of an amino acid selected especially from the group L-lysine, L-methionine, L-threonine, L-isoleucine, L-glutamate, L-glutamine, L-proline, L-arginine, L-citrulline and L-ornithine especially L-lysine.

The methods of culturing the L-amino acid producing strains thus obtained are conventional, and are similar to the methods for the cultivation of known L-amino acids producing microorganisms.

The culture medium employed can be a conventional medium containing carbon sources, nitrogen sources, and organic ions and, when required, minor organic nutrients such as vitamins and amino acids. Examples of suitable carbon sources include glucose, sucrose, lactose, starch hydrolysate and molasses. Gaseous ammonia, aqueous ammonia, ammonia salts and other nitrogencontaining materials can be used as the nitrogen source.

Cultivation of the transformed organisms containing the vehicle carrying the *ppc* gene is conducted under aerobic conditions in which the pH and the temperature of the medium are adjusted to a suitable level and continued until the formation of L-amino acid ceases. In a preferred embodiment a transformed *Corynebacterium glutamicum* is used, selected from the group of those having the identifying characteristics of *Corynebacterium glutamicum* DSM 4697 deposited under the provisions of the Budapest Treaty on

July 8, 1988 at Deutsche Sammlung von Mikroorganismen (DSM).

The amino acids, which accumulate in the culture medium, can be recovered by conventional procedures.

By the methods of the present invention, L-amino acids, especially L-lysine, can be produced in higher yields than has been achieved in previously known methods using artificial mutants of *Brevibacterium* and *Corynebacterium*.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples, which are provided herein for purposes of illustration only, and are not intended to be limiting, unless otherwise specified.

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## 1. Isolation of the *Corynebacterium glutamicum* ATCC13032 *ppc* gene.

### 1.1 Construction of genomic bank of *C. glutamicum* ATCC13032

Total DNA from *C. glutamicum* ATCC13032 was isolated as described by Chater et al. (Curr. Topics Microb. Immunol. 96, 69 pp (1982)) and partially digested with *Sau*3AI. Fragments ranging in size from 4-20 kb were purified from low melting temperature agarose. The DNA solution, was dialyzed against 2 L of TE buffer (Tris 10 mM, EDTA 1 mM). 2 µg of size fractionated chromosomal DNA was ligated using T4 DNA Ligase with 1 µg of plasmid pUC18 (Yanish-Perron, C. et al. (1985) Gene 33, 103 pp) which had been digested with *Bam*HI and treated with alkaline phosphatase.

*E. coli* NM522 (Gough, J.A. and Murray, N.E. (1983) J. Mol. Biol. 166, 1 pp) was transformed according to Hanahan (J. Mol. Biol. 166, 557 pp (1983)) with the ligation mixture and transformants were selected at 37°C on LB agar plates (Davis, R.W. et al. (1980) Advanced Bacterial Genetics, Cold Spring Harbor Laboratory) containing ampicillin (100 µg/ml) and 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal, 50 µg/ml). 80 % of clones were colourless on X-gal plates, indicating the presence of inserted DNA. To estimate more precisely the efficiency of cloning, plasmid DNA from 46 colourless clones was examined by restriction enzyme digestion : 7 % of clones contained no inserts; 41 % of clones contained small inserts with an average size of 0.5 kb; 52 % of clones contained inserts ranging in size from 1.35 kb to 8.5 kb with an average size of 5 kb.

In total 10<sup>4</sup> clones were obtained upon transformation of strain NM522. These clones were pooled in 4 families (CgSA, CgSB, CgSC and CgSD) and plasmid DNA was prepared by CsCl-EtBr density gradient centrifugation.

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### 1.2 Cloning of the *ppc* gene

Competent cells of *E. coli* XH11 (Mountain, A. et al. (1984) Mol. Gen. Genet 197, 82 pp) were transformed with 5 x 200 ng of each of the families of the *C. glutamicum* gene bank. Transformation mixes were spread on M9 agar (Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory) plus arginine (50 µg/ml) and ampicillin (100 µg/ml) and on LB plus ampicillin (100 µg/ml). A transformation frequency of 10<sup>4</sup>/µg was obtained on LB plus ampicillin. 108 clones were isolated on M9 agar containing arginine and ampicillin from the transformation with family CgSD. Restriction enzyme digestion of isolated DNA indicated that all clones contained the same plasmid. The plasmid (pTG1200) consisted of pUC18 plus an insert of approximately 5 kb. A restriction map of the insert of pTG1200 is shown in Figure 1. Retransformation of XH11 by pTG1200 led to complementation of the *ppc* mutation. Southern hybridisation (Maniatis, T. et al. (1982) Molecular Cloning, Cold Spring Harbor Laboratory) confirmed that chromosomal DNA of *C. glutamicum* ATCC13032 had been cloned in pTG1200.

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### 1.3 Localization of the *ppc* gene on the cloned DNA fragment.

To better localize the *ppc* gene, subcloning experiments were undertaken. Digestion of pTG1200 with *Sall* generates an internal fragment of 3.5 kb (Figure 1). pTG1200 digested with *Sall* was ligated with *Sall* cut pBR322 (Bolivar, F. et al. (1974) Gene 2, 95 pp). To eliminate religated pTG1200 molecules the ligation mixture was digested with *Xba*I. *E. coli* XH11 was transformed with the ligation mixture and plated on M9 agar containing arginine and ampicillin. Plasmid DNA of complemented clones was examined and found to

consist of pBR322 plus the 3.5 kb Sall fragment of pTG1200.

The constructed plasmid was designated pTG1201 (Figure 2) and the corresponding strain XH11 pTG1201.

#### 1.4 Measurement of enzyme activity in *C. glutamicum* ATCC13032 and in *E. coli* clones carrying a recombinant plasmid containing the DNA fragment with the *C. glutamicum* ppc gene.

Phosphoenolpyruvate carboxylase activity was assayed in *C. glutamicum* ATCC13032 and in *E. coli* strain XH11 pTG1201. *E. coli* strain MM294 (Hanahan, D. (1983) J. Mol. Biol. 166, 557 pp) was used as a positive control and strain XH11 as negative control. *C. glutamicum* ATCC13032 was cultivated in MMYE medium (Katsumata, R. et al. (1984) J. Bact. 159, 306 pp) and *E. coli* strains in M9 medium supplemented with arginine (50 µg/ml) and ampicillin (100 µg/ml) in the case of XH11/pTG1201, with thiamine (200 µg/l) in the case of MM294 and with sodium succinate (5 g/l) and arginine (50 µg/ml) in the case of XH11. The growth conditions were 37°C and 150 rpm for *E. coli* strains and 30°C and 150 rpm for *C. glutamicum*.

The cultures were harvested by centrifugation at the beginning of the stationary phase of growth and washed three times with a buffer composed of 100 mM Tris/HCl pH 7.5 and 1 mM DTT. Disruption of the cells was performed in a glass bead mill (MSK-Homogenisator; B. Braun Melsungen, FRG). PEP carboxylase activity in the clear supernatants was determined after extensive dialysis against 100 mM Tris/HCl pH 7.5; 0.8 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 1 mM DTT. The modified malate dehydrogenase coupled assay (Ozaki, H. and Shio, J. (1969) J. Biochemistry 66, 297 pp) was used and NADH disappearance was followed photometrically at 340 nm. The assay mixture included the following components: 6 mM PEP; 10 mM NaHCO<sub>3</sub>; 100 mM Tris/HCl pH 7.5; 0.15 mM NADH; 2 U/ml malate dehydrogenase (pig heart); 3.3 mM MnSO<sub>4</sub> and PEP carboxylase preparation in a final volume of 1 ml. Unspecific NADH decomposition was measured before starting the reaction by addition of Mn<sup>2+</sup>. Protein concentration was determined by the methods of Lowry et al. (Lowry, O.H. et al. (1951) J. Biol. Chem. 193, 265 pp) or Bradford et al. (Bradford, M.M. (1976) Analyt. Biochem. 72, 248 pp).

The data shown in Table 1 confirm that the 3.5 kb Sall DNA fragment cloned from *C. glutamicum* ATCC13032, contained in plasmid pTG1201, encodes the phosphoenolpyruvate carboxylase gene.

In particular, the effect of acetyl-CoA known to stimulate phosphoenolpyruvate carboxylase from *E. coli* (Izui, K. et al. (1981) J. Biochem. 90, 1321 pp) and *Brevibacterium flavum* (Ozaki, H. et al. (1969) J. Biochem. 66, 297 pp) was investigated. From the results shown in Table 1 it is evident that phosphoenolpyruvate carboxylase from *C. glutamicum* ATCC13022, either produced in its original host or as a result of cloning into *E. coli* to form strain XH11/pTG1201, is not stimulated by acetyl-CoA under the assay conditions described above.

Table 1

Strains	Phosphoenolpyruvate carboxylase activity (U/mg protein)	
	without acetyl-CoA	in the presence 0.2 mM acetyl-CoA
<i>C. glutamicum</i> ATCC13032	0.226	0.225
<i>E. coli</i> MM294	0.035	0.158
<i>E. coli</i> XH11/pTG1201	1.010	1.090
<i>E. coli</i> XH11	0.0	0.0
Phosphoenolpyruvate carboxylase activity in dialyzed homogenates obtained from <i>C. glutamicum</i> and from different <i>E. coli</i> strains.		

#### 2. Determination of the nucleotide sequence of the ppc gene of *C. glutamicum* ATCC13032.

The nucleotide sequence of the entire 5 kb insert of pTG1200 was sequenced using a shot gun

approach (Messing, J. et al. (1981) Nucleic Acids Res. 9, 309 pp). The SmaI site of pTG1200 was replaced by an XbaI site by cloning of a blunt ended oligomer (GTGTCTACAGTG) to generate a new plasmid called pTG1202. 10 µg of the 5.0 kb XbaI insert of pTG1202 was purified from low-melting-temperature agarose. The fragment was re-circularised using T4 DNA ligase, randomly fragmented by sonication and finally blunt ended using Klenow polymerase in the presence of 2 mM of each dATP, dGTP, dCTP and dTTP. Fragments ranging from 300-800 bp in size were isolated from low melting point agarose and ligated with SmaI digested M13mp8 phage (Messing, J. and Vieira, J. (1982), Gene 19, 269-276). 160 clones were sequenced by the chain termination method (Sanger, F. et al. (1977) Proc. Natl. Acad. Sci. USA 74, 5463 pp) and over-lapping clones identified by computer-aided analysis (DNASTAR, Inc., 1801 University Ave., Madison, WI 53705, USA). The entire sequence of the inserted DNA fragment contained in pTG1202 bearing the ppc gene of C. glutamicum ATCC13032 is shown in Figure 3.

The entire sequence which has been determined is 4885 bp in length. A search for possible protein encoding regions has revealed a long open reading frame (ORF) showing homology to ppc gene sequences from E. coli (Fujita, N. et al. (1984) J. Biochem. 95, 909 pp) and other organisms (Katagiri, F. et al. (1985) Gene 38, 265 pp and Izui, K. et al. (1986) Nucleic Acids Res. 14, 1615 pp) indicating that this ORF encodes the PEP carboxylase gene. This ORF is contained within the 3.5 kb Sall DNA fragment extending between coordinates 852 and 4077, cloned in pTG1201. Sequence analysis identified two possible start sites of translation (coordinates 921 and 906) giving rise to a protein product of either 919 or 924 amino acids respectively.

## 2.1 Determination of the N-terminal sequence of the PEP carboxylase protein.

In order to precisely identify the initiation codon of the ppc gene product, the N-terminal amino acid sequence of the PEP carboxylase protein was determined.

Purification of PEP carboxylase from C. glutamicum ATCC13032 was performed after disruption of the cells using a glass bead MSK-Homogenisator. After precipitation of nucleic acids by 0-3% Streptomycin sulphate, a fractionated  $(\text{NH}_4)_2 \text{SO}_4$  (0-50% and 50-70%) precipitation was performed. The redissolved pellet from the 50-70%  $(\text{NH}_4)_2 \text{SO}_4$  precipitation was dialyzed against starting buffer (20 mM Tris/HCl pH 7.5; 100 mM  $(\text{NH}_4)_2 \text{SO}_4$ ; 1 mM DTT) and further purified by ion exchange chromatography on Q Sepharose fast flow. Gel-filtration on Sephacryl S300 superfine was then performed with the concentrated PEP carboxylase fractions: elution was with stabilizing buffer (100 mM Tris/HCl pH 7.5; 800 mM  $(\text{NH}_4)_2 \text{SO}_4$ ; 1 mM DTT). Finally, samples were processed through an affinity chromatography step on Blue Sepharose, eluted using L-aspartate (70 mM) and dialyzed against stabilizing buffer to remove aspartate. The homogenous PEP carboxylase fractions - as proved by SDS-PAGE - were then concentrated by a factor of about four using a speed-vac concentrator at 5°C.

Desalting of enzyme fractions (1 ml containing about 25 µg of protein) was performed by dilution with 5 ml TRIS/HCl 100 mM pH 7.5. Samples were then concentrated to a final volume of 1.5 with a 8400 Amicon concentrator using a YM30 membrane (2.5 cm diameter). This procedure was repeated twice. The concentrated solution was divided into three (2 ml) Eppendorf tubes. Protein precipitation was then performed at -80°C 48 h after addition of ethanol (2 ml) in each Eppendorf tube. Pellets were pooled, washed twice with -80°C cooled ethanol/water (80/20 V/V). Samples were loaded on a precycle filter for sequencing after solubilization in formic acid. Sequencing was performed on an Applied Biosystem 470A protein sequencer with an on-line 120A PTH-analyser.

The obtained sequence is the following:  
Thr<sup>1</sup>-Asp-Phe-Leu-Arg<sup>5</sup>-Asp-Asp-Ile-Arg-Phe-<sup>10</sup>-Leu-Gly-Gln-Ile -Leu<sup>15</sup>

This result shows that PEP carboxylase is encoded by the ORF of 919 amino acids stretching between the ATG codon at position 921 and the TAG codon at position 3678. The initiation codon lies about 14 base pairs downstream from a putative Shine-Dalgarno sequence (coordinates 900-908, Figure 3).

## 3. Cloning and expression of the ppc gene from C. glutamicum ATCC13032 in C. glutamicum ATCC13032.

### 3.1 Construction of the C. glutamicum/E. coli shuttle vector pZ1.

The structure of the plasmid shuttle vector pZ1 is shown in figure 4. It was constructed from E. coli



vector pACYC177 (Chang, A.C.Y. and Cohen, S.N. (1978) J. Bact. 134, 1141 pp) and from *C. glutamicum* plasmid pHM1519 (Miwa, K. et al. (1984) Agric. Biol. Chem. 48, 2901 pp) as disclosed in Deutsche Patentanmeldung 3737729.9 *E. coli* strain DM272-3 containing plasmid vector pZ1 was deposited at the Deutsche Sammlung von Mikroorganismen under DSM4242.

### 3.2 Cloning of the *ppc* gene onto the *C. glutamicum*/*E. coli* shuttle vector pZ1.

Plasmid pTG1200 was isolated from *E. coli* XH11/pTG1200 and partially digested with *Sac*3A and plasmid pZ1 isolated from DM272-3 (= DSM4242) was linearized with *Bgl*III. Both DNAs were mixed, ligated with T4 DNA Ligase and the ligation mixture used to transform *E. coli* XH11. Transformants were selected on M9 agar containing arginine (50 µg/ml) and Kanamycin (10 µg/ml). Plasmid-DNA from one of the transformants called XH11/pDM2 was isolated and characterized by restriction mapping. The structure of pDM2 is shown in Figure 5. Enzyme measurements revealed that strain XH11/pDM2 had a phosphoenolpyruvate carboxylase activity which was not stimulated by acetyl-CoA (Table 2) as was shown in Table 1 for strain XH11/pTG1201.

Table 2

Strains	Phosphoenolpyruvate carboxylase activity (U/mg protein)	
	without acetyl-CoA	in the presence of 0.2 mM acetyl-CoA
<i>C. glutamicum</i> ATCC13032	0.226	0.225
<i>E. coli</i> XH11	0.0	0.0
<i>E. coli</i> XH11/pDM2	0.261	0.272
Phosphoenolpyruvate carboxylase activity in dialyzed homogenates obtained from <i>C. glutamicum</i> ATCC13032 and from <i>E. coli</i> strains XH11 and XH11/pDM2.		

### 3.3 Construction of the *C. glutamicum* vector PCV22.

Plasmid pHM1519 (Miwa, K. et al. (1984) Agric. Biol. Chem. 48, 2901 pp) isolated from *C. glutamicum* ATCC13058 was cut with *Bgl*III and ligated to the pUCB (Vieira, J. and Messing, J. (1982) Gene 19, 259 pp) derivative pSVB21 (Arnold, W. et al. Gene (1988) 70, 171 pp) cut with *Bam*HI. The ligation mixture was used to transform *E. coli* strain JMB3 (Vieira, J. and Messing, J. (1982) Gene 19, 259 pp) and transformants were selected on LB agar containing ampicillin. Plasmid DNA was isolated from one of the transformants and called pECS100.

The Kanamycin resistance gene of Tn5 (Jorgensen, R.A. et al. (1979) Mol. Gen. Gen. 177, 65 pp) was isolated as an *Xho*I-*Sal*I DNA fragment from a pACYC184 (Chang, A.C.Y. and Cohen, S.N. (1978) J. Bact. 134, 1141 pp) derivative carrying Tn5 and inserted into the *Sal*I site of pECS 100 to form pECS300 which was transferred to *C. glutamicum* ATCC13032 by transformation (Yoshihama, M. et al. (1985) J. Bact. 162, 591 pp).

Plasmid pECS300 was isolated from strain ATCC13032/pECS300 digested with *Sma*I, religated and used to transform *C. glutamicum* ATCC13032. Plasmid was isolated from one of the transformants, characterized by restriction mapping and called pECS330.

The *E. coli* replicon including the  $\beta$ -lactamase resistance gene was deleted from pECS330 by digestion with *Hind*III, religation and transformation to form the *C. glutamicum* vector pCV20.

Plasmid pCV20 was digested with *Sma*I and ligated with the 0.322 Kb *Pvu*II fragment of pUC19 carrying the *E. coli* *lacZ* promoter and the multiple Cloning site (Yanisch-Perron, C. et al. (1985) Gene 33, 103 pp). *C. glutamicum* ATCC13032 was transformed with the ligation mixture. Plasmid DNA was isolated from one of the transformants, named pCV22 and its structure confirmed by restriction mapping. The structure of pCV22 including its construction as described above is shown in figure 6.

### 3.4 Cloning of the ppc gene onto the C. glutamicum vector pCV22.

Plasmid pDM2 was isolated from E. coli strain XH11/pDM2 by ethidium bromide CsCl density gradient centrifugation and used to transform C. glutamicum ATCC13032 as described by Yoshihama et al. (J. Bact. 162, 591 pp (1985)). Plasmid DNA was isolated from one of the transformants and shown to have the structure of pDM2.

Plasmid pCV22 was isolated from ATCC13032/pCV22 cut with Sall and treated with calf intestine alkaline phosphatase. Plasmid pDM2 was cut with Sall and SmaI. Both DNA's were mixed, ligated with T4 DNA Ligase and the ligation mixture used to transform C. glutamicum ATCC13032 (Yoshihama, M. et al. (1985) J. Bact. 162, 591 pp). Plasmid DNA designated pDM6 was isolated from one of the transformants and characterized by restriction mapping. The structure of pDM6 is shown in Figure 7.

### 3.5 Measurement of enzyme activity in C. glutamicum clones carrying a recombinant plasmid containing the DNA fragment with the ppc gene.

Phosphoenolpyruvate carboxylase was measured in C. glutamicum strains ATCC13032/pCV22, ATCC13032/pDM2 and ATCC13032/pDM6. The result is shown in Table 3.

Table 3

<u>C. glutamicum</u> strains	Phosphoenolpyruvate carboxylase activity (U/mg protein) in the absence of acetyl-CoA
ATCC13032/pCV22	0.250
ATCC13032/pDM2	0.240
ATCC13032/pDM6	0.996
Phosphoenolpyruvate carboxylase activity in dialyzed homogenates obtained from different <u>C. glutamicum</u> strains.	

### 4. Effect of plasmid pDM6 on PEP carboxylase activity and on the lysine excretion of the lysine excreting strain C. glutamicum DM58-1.

C. glutamicum strain DM58-1 is a derivative of strain ATCC13032, resistant to 50 mM of the L-lysine analogue S-2-aminoethyl-DL-cysteine, obtained after conventional N-methyl-N-nitro-N-nitrosoguanidine mutagenesis.

Plasmid pDM6 and as a control plasmid pCV22 were introduced into C. glutamicum DM58-1 to give strains DM58-1/pDM6 and DM58-1/pCV22. Strain DM58-1/pDM6 was deposited at the Deutsche Stammsammlung von Mikroorganismen under DSM4697. The results obtained by measuring the specific PEP carboxylase activity and the concentration of excreted lysine as well as sucrose consumption are shown in Table 4.

The stimulating effect of the elevated level of PEP carboxylase exerted by the cloned ppc gene of plasmid pDM6 on the concentration of lysine excreted and in particular on the yield which is the amount of lysine formed per amount of sucrose consumed is evident.

Strains	PEP carboxylase activity (U/mg protein)	Concentration of L-lysine excreted (g/l)	Yield g L-lysine g sucrose
<u>C. glutamicum</u> DM58-1	0.275	16.1	0.170
<u>C. glutamicum</u> DM58-1/pCV22	0.240	15.9	0.173
<u>C. glutamicum</u> DM58-1/pDM6	0.955	17.9	0.198

**Table 4: Effect of increased level of PEP carboxylase on lysine excretion by C. glutamicum.**

The cultivation was carried out in 100 ml-flasks with indentations containing 10 ml of a medium composed of 12 g/l ammoniumsulfate, 240 g/l molasses, 60 ml/l soy bean protein hydrolysate and 10 g/l CaCO<sub>3</sub>. In the case of strains DM58-1/pCV22 and DM58-1/pDM6 the medium contained 20 µg/ml kanamycin. Incubation was carried out for 48h at 30°C and at 300 rpm. After completion of the cultivation lysine in the culture supernatant was quantitatively determined by amino acid analyzers using ion exchange chromatography and ninhydrin detection. Sucrose was quantitatively determined by an enzymatic assay using invertase coupled to hexokinase and glucose-6-phosphat dehydrogenase (Technicon Application Note AAll: Saccharose and Glucose).

#### 5. Effect of plasmid pDM6 on the threonine and isoleucine excretion of strain B. flavum DM368-2

B. flavum strain DM368-2 is a derivative of strain ATCC14067, resistant to 4 mg/ml of the threonine analogue  $\alpha$ -amino- $\beta$ -hydroxy-valeric acid, obtained after conventional N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis.

Plasmid pDM6 was isolated from C. glutamicum DM58-1/pDM6 (= DSM4697) and introduced into B. flavum DM368-2 to give strain DM368-2/pDM6. Strain DM368-2 was deposited at the Deutsche Sammlung von Mikroorganismen under DSM 5399. The effect of plasmid pDM6 on the concentration of excreted L-threonine and L-isoleucine as well as sucrose consumption are shown in Table 5.

The stimulating effect of the cloned ppc gene contained in plasmid pDM6 on the concentration of threonine and isoleucine excreted and in particular on the yield which is the amount of amino acid formed per amount of sucrose consumed is evident.

5	Strains	Amino acid excreted	Concentration of amino acid excreted g/l	Yield g amino acid g sucrose
10	<i>B. flavum</i> DM368-2	L-threonine L-isoleucine	3.32 0.92	0.0324 0.0090
15	<i>B. flavum</i> DM368-2/pDM6	L-threonine L-isoleucine	3.77 1.08	0.0370 0.0106

Table 5: Effect of pDM6 on threonine and isoleucine excretion by *B. flavum*.

The cultivation was carried out as described under 4.

Figure 1: Restriction map of the insert of pTG1200.

Figure 2: Restriction map of pTG1201.

Figure 3: Nucleotide sequence of the DNA fragment inserted into pTG1200 containing the ppc gene.

Figure 4: Restriction map of pZ1.

Figure 5: Restriction map of pDM2.

Figure 6: Construction and restriction map of pCV22.

Figure 7: Restriction map of pDM6.

### Claims

1. A DNA fragment isolated from a *C. glutamicum* strain containing a genetic sequence comprising information coding for the production of a protein having the activity of phosphoenolpyruvate carboxylase (PEPC).

2. The DNA fragment of Claim 1, which consists essentially of a fragment of 3422 base pairs flanked at its termini by Sal I restriction sites.

3. The DNA fragment of Claim 1 or 2, which consists of 2757 base pairs coding for the structural gene of PEPC.

4. The DNA fragment of any of the Claims 1, 2 or 3 coding for the production of PEPC, which shows the N-terminal amino acid sequence Thr<sup>1</sup> - Asp - Phe - Leu - Arg<sup>5</sup> - Asp - Asp - Ile - Arg - Phe<sup>10</sup> - Leu - Gly - Gln - Ile - Leu<sup>15</sup>.

5. The DNA fragment of any of the Claims 1, 2, 3 or 4 isolated from *Corynebacterium glutamicum* ATCC 13032.

6. A vehicle capable of replication in *Corynebacterium* or *Brevibacterium* containing the DNA fragment of any of the Claims 1, 2, 3, 4 or 5.

7. The expression vehicles of Claim 6, which are the plasmids pDM2 or pDM6.

8. A host bacterium belonging to the genus *Corynebacterium* or *Brevibacterium* containing the vehicle of the Claims 6 or 7, optionally producing the amino acid of interest.

9. A *Corynebacterium* which is selected from the group of those having the identifying characteristics of DSM 4697.

10. A *Brevibacterium* which is selected from the group of those having the identifying characteristics of DSM 5399.

11. A method of producing L-amino acid selected from L-lysine, L-methionine, L-threonine, L-isoleucine, L-glutamate, L-glutamine, L-proline, L-arginine, L-citrulline and L-ornithine by fermentation which comprises culturing in an appropriate medium a bacterium of Claims 8, 9 or 10 and recovering the L-amino acid from said medium.

12. A method of producing L-lysine by fermentation according to Claim 11, which comprises culturing

the bacterium of the Claims 8 or 9 and recovering L-lysine.

13. A method of producing L-isoleucine and L-threonine by fermentation according to claim 11, which comprises culturing the bacterium of the Claims 8 or 10 and recovering L-isoleucine and L-threonine.

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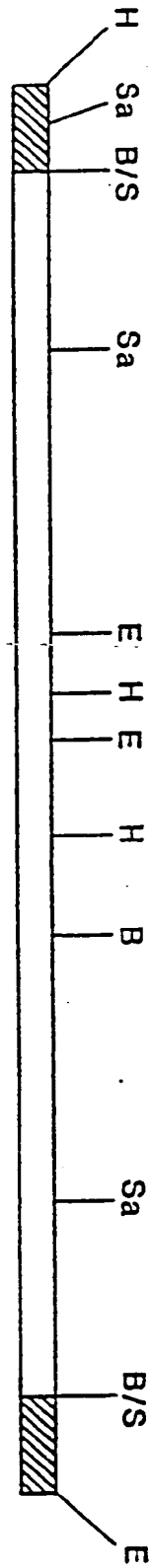
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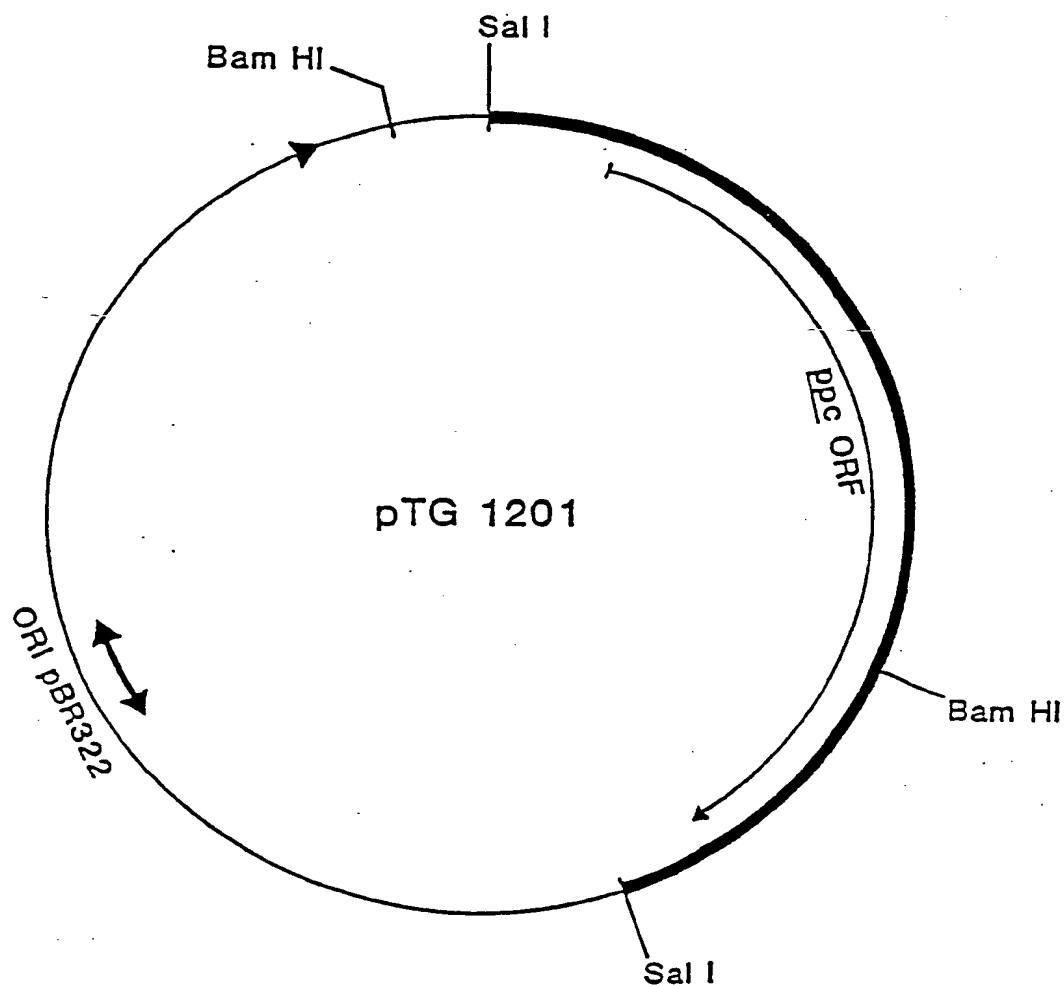
55

1 Kb



**FIGURE 1 : RESTRICTION MAP OF THE INSERT OF PLASMID pTG1200**

Legends to symbols: Open bar: 5 kb insert; dashed bars: polylinker of pUC18 vector. H, HindIII; Sa, Sma; B, BamHI; E, EcoRI; B/S, BamHI/Sau3A hybrid.



**FIGURE 2 : RESTRICTION MAP OF PLASMID pTG1201**

Thick line: 3.5 kb SalI fragment bearing the ppc ORF.  
Thin line : pBR322 vector. ► indicates position and orientation of the promoter of the tetracycline resistance determinant.

The predicted amino acid sequence corresponding to phosphoenolpyruvate carboxylase is indicated underneath the corresponding DNA sequence.

[illegible]



[illegible]



TGCTGATTCTGCCATCGAGAGCTTTGGATTCAACCTTTACGCCACTGGATCTGCGCCAAACCTCCGAAAGCTACGAGGACGTCCTCACCAGGCTTTTCGA  
 l i s a i e s f g f n l y a l d l r q n s e s y e d v l t e l f e  
 -----  
 ACGGCCCCAAGTCACCGCAAACCTACCGCGGAGCTGTCTGAAGCAGAGAAGCTTGAGGTGCTGCTGAAGGAACCTGGCAGAGCCTCGTCCGCTGATCCCGCAC  
 r a q v t a n y r e l s e a e k l e v l l k e l r s p r p l i p h  
 -----  
 GGTTCAGATGAATACAGCGAGGTCACCGACCGGAGCTCGGCATCTTCGGCACCGGCTCGGAGGCTGTTAAGAAATTCGGGCCACGGATGGTGCTCACT  
 g s d e y s e v t d r e l g i f r t a s e a v k k f g p r m v p h c  
 -----  
 GCATCATCTCCATGGCATCATCGGTACCGCATGTGCTCGAGCGCGATGGTGTTCAGGAATTCGGACTCATCGCAGCCAAACGGCGGACAAACCCACGCGG  
 i i s m a s s v t d v l e p m v l l k e f g l i a a n g d n p r g  
 -----  
 CACCGTCGATGTCATCCACACGTTCGAAACCATCGAAGATCTCCAGGCGGCGCGCGGAATCCTCGACGAACCTGTGGAAATTCATCTCCTACCGCAACTAC  
 t v d v i p l f e t i e d l q a g a g i l d e l w k i d l y r n y  
 -----  
 CTCTCTCGAGCGGACAAACGTCACGGAAGTCATGCTCGGTACTCCGATTCCAACAAGGATGGCGGATATTTCTCCGCAAACTGGGCGCTTTACGACGCGG  
 l l l q r d n v q e v m l g y s d s n k d g g y f s a n w a l y d a e  
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Figure 3 continued (e)

Figure 3 continued (f)

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ATGAGTCCCTGGCCATTTTTCACCTCAGTGTGGATAACATGGCTCAGGTGATGTCCAGGCAGAGCTGGGTTTGGCAAGCTCTACGCAGACCTGATCCC
e s w p f t s v l d n m a q v m s k a e l r l a k l y a d l i p
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
AGATACGGAAGTAGCGGAGGAGTCTATTCCGTCATCCGGGAGGAGTACTTCCCTGACCAGGAAGATGTTCTGGGTAATCACCAGGCTCTCATGATCTGCTT
d t e v a e r v y s v i r e e y f l t k k m f c v f t g s d d l l
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
GATGACAACCCACTTCTGGCAGGCTCTGTCCAGGCGGATACCCCTACCTGCTCCACTCAACGTTGATCCAGGTAGAGATGATGCCAGCGCTACCGAAAG
d d n p l l a r s v q r r y p y l l p l n v i q v e m m r r y r k g
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
GCGACCAAGCGAGCAAGTGTCCCGCAACATTTCAGCTGACCATGAACGGTCTTTCACCTGGCTGGCAACCTCCGGCTAGTCCAGCGGGCTGGGTAGTAC
d q s e q v s r n i q l t m n g l s t a l f n s g .
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
TCGTGTACTGTCTAAAGTTATTCCGAATCAGGTGGGCATAAGGTTACCTGGTCTCAACGGCAAGGAACATTTTCCACATGGCATTGACGCTTC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
AAATCATCTCTGTCGCCAGCCTGCCTCATGACGGTTTTCGCTCTGTCGACAAAGGGCGGGGACCTCCAGCCCTCTTCGGTGGCGGTGTGCA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
GTCCAATCTTTCGGGCTCCACTGTTGTGAAAGAACCTGGATCGGCTCACCATTTTGGTTGGCGTTATCTGGATTGTGTGCATTGTGCGCACTCAACCTC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

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3400

3500

3600

3700

3800

3900

4000

Figure 3 continued (g)

ATCCAGACTTATTCATAAGACACGAGCTTAAAAAGAGCGGTTCCTTTTCATAGGGAGCGGCTTTTGGGTTTGTGCGACCTGTTGTCCTCCACACTGT  
 4100  
 TCCTCGGTGTGCACCTTCGACACCAAGATTTCGGCAAGTGGTGGTCAAAATTGGAAAATCTTGGTGCCTAAATTCACATACATTCCTCAATTTTCCCTAAGG  
 4200  
 ACATCTTTAAAGGGGAACCTGTTCCCGACGGAACGTGGAGTCTATAAAACCGCAGGTTAAACGGCTGCCATAGGGGATTTTTCGGCTGGGGAGACGTGGT  
 4300  
 GTAAGTGGGGTTAAAAACGTGACCTTCGTTATAAAAAACAGAAATCTATAGAACGATAGGTAAAACTGGACTAGGTTTATCTATAGCGGAATAGAAAT  
 4400  
 ACTCCGCTCGACAGCATCACTTAGCTGAAAGGCCTTTAACATGGACCCCTCAGATCTAGCCTGGATTCTCGCAGCTTTTGGGTGGTAAGCCTGATGTTT  
 4500  
 CCGGATTGTCCCTGCTACGGGGCGCATGCTGGGTGGGCAACACGTTCTTAACACGTTCAATGATGGTTATGAGCTCACTTGGAAATCATCAGCCTTGTGT  
 4600  
 ACATCATTTATGGACACGGACTTGTCTTAGGAAACTCCATCGGTGGGTGGGGAATTATCGGAATCCCTTGAATACTTCCGCTTCCGCAACATTATGGA  
 4700  
 AGATGACGGCACCGGAGACCTCATGTGGCCGGCTTCTACATTCTGTTCCGTGCAATCTCACTGGCAGCTTGTTCATCTGGTGCAGCGGGCGGCATGCGC  
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 4885

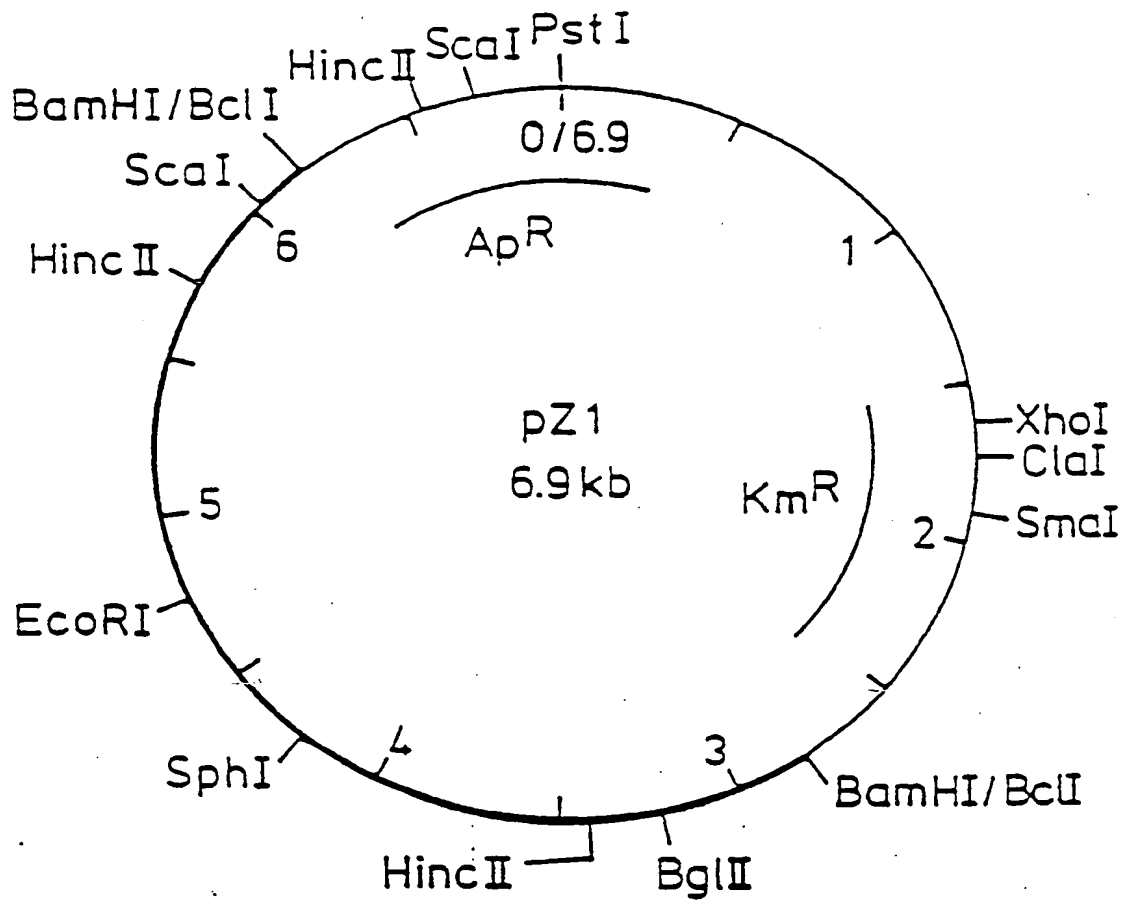
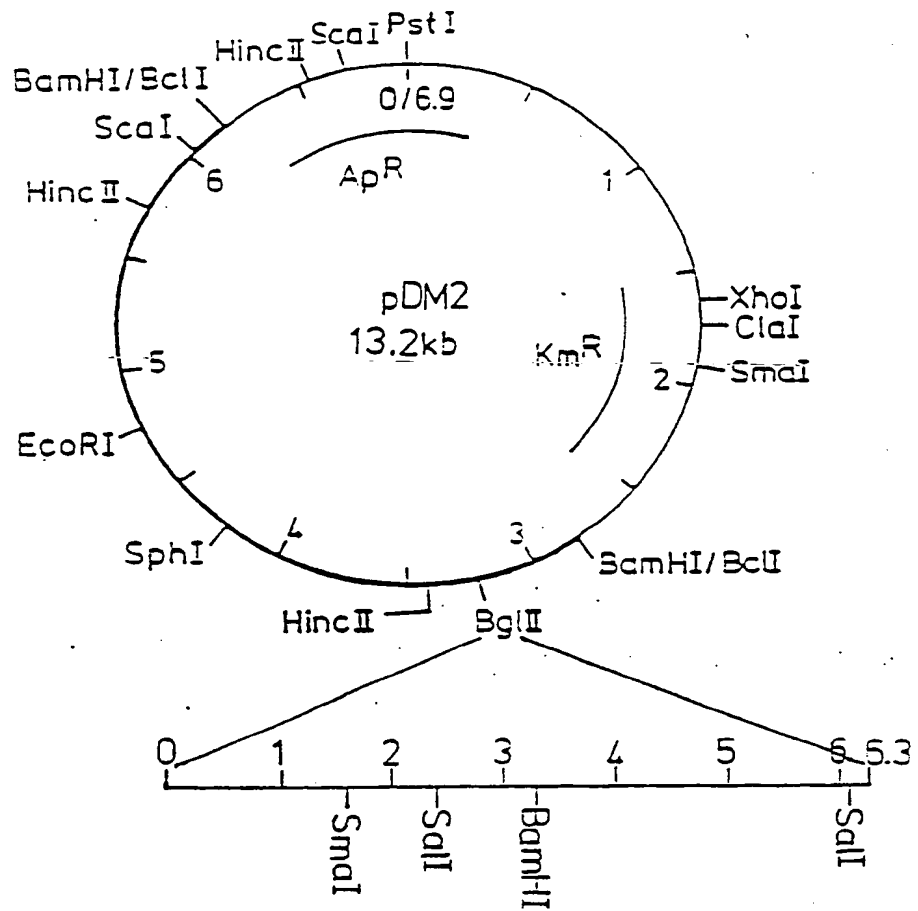


Figure 4: Restriction map of pZ1.

New eingetragen/RCS  
New eingetragen/RCS



**Figure 5:** Restriction map of pDM2.



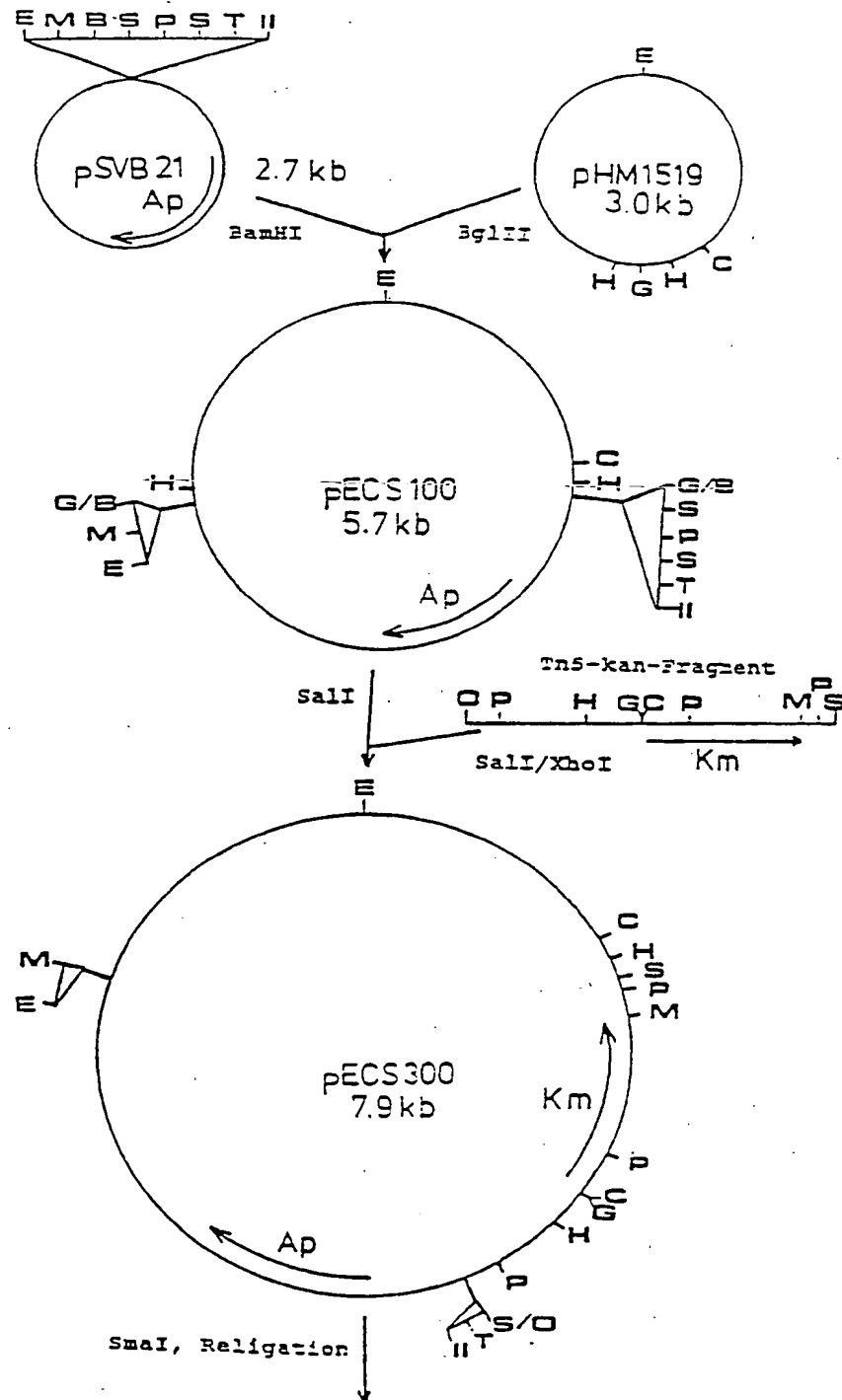


Figure 6:

SmaI, Religation

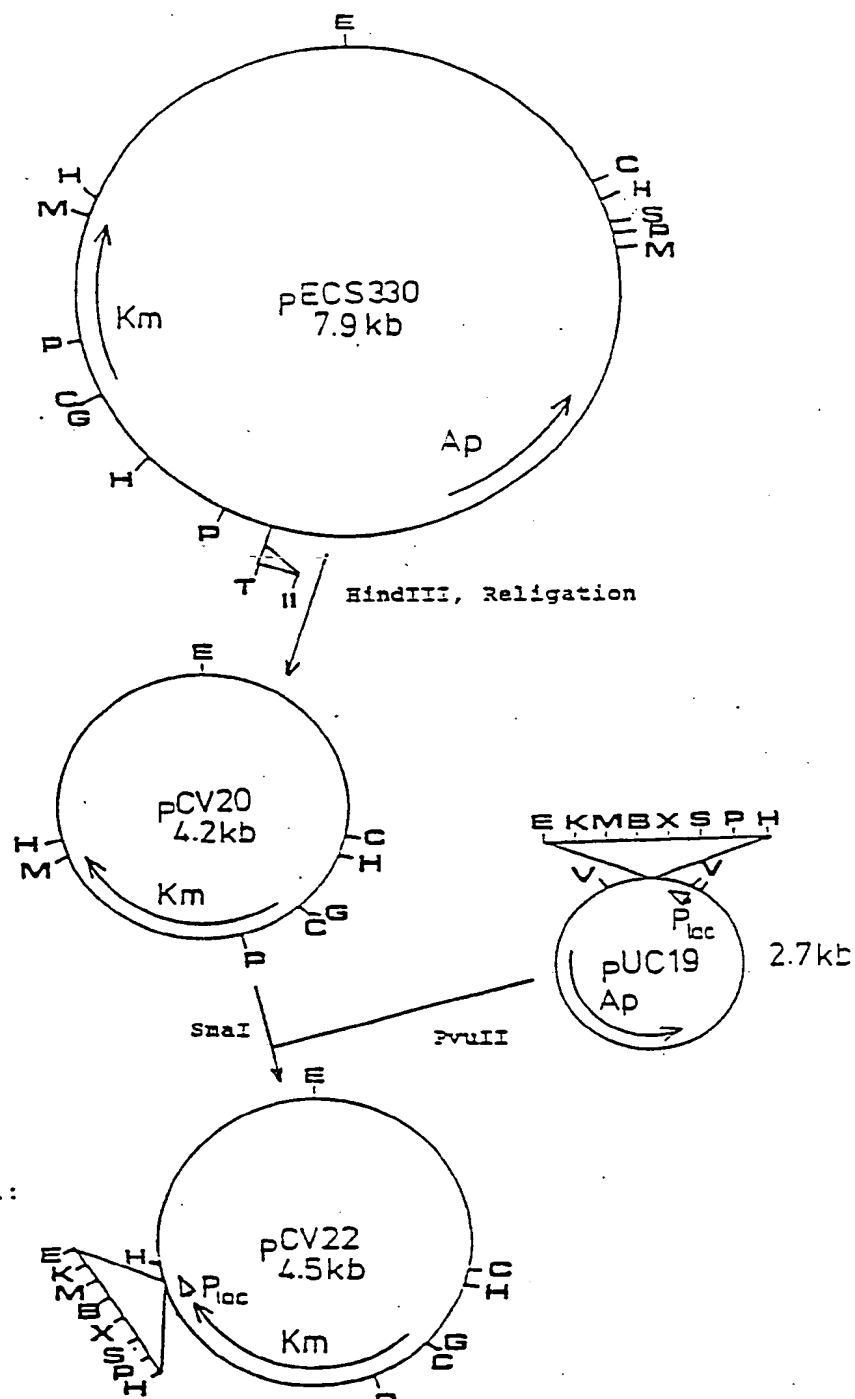


Figure 6 contd.:

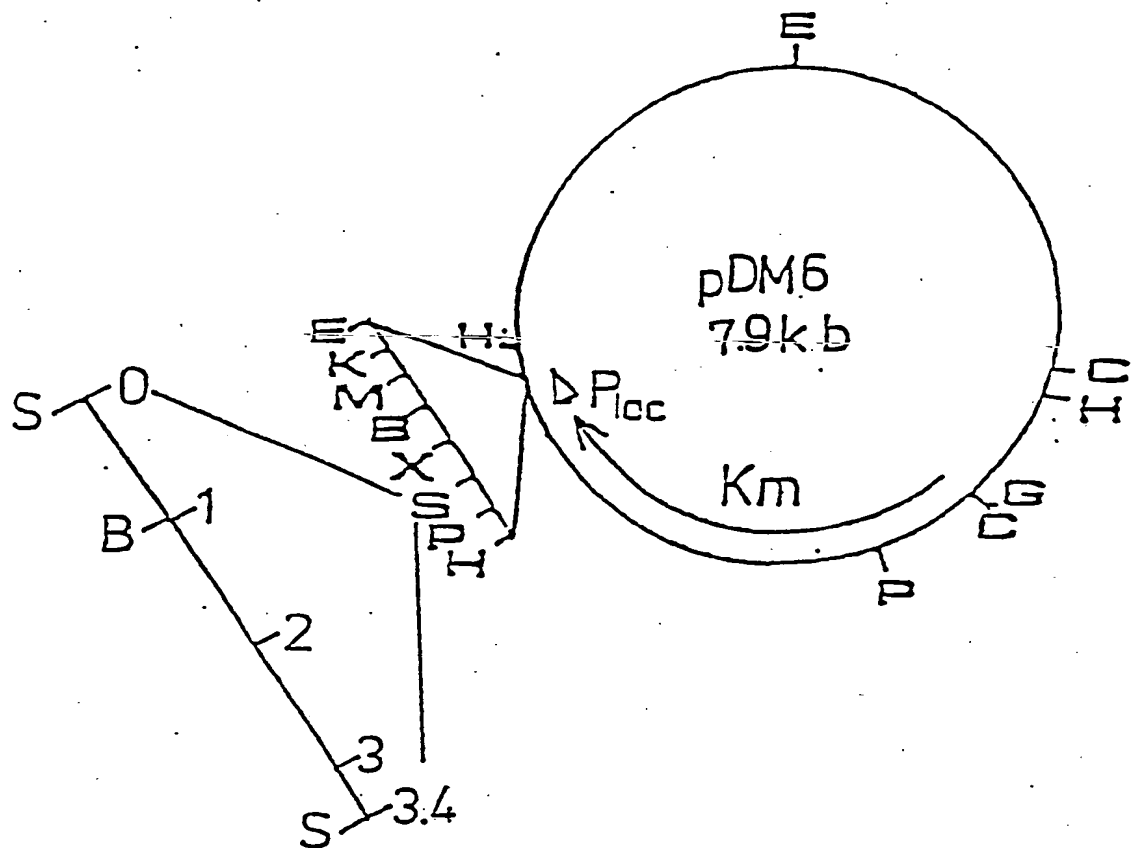
Figure 6 contd.: Construction and restriction map  
of pCV22. Legends to symbols:

B : BamHI  
 C : BclI  
 E : EcoRI  
 G : BglII  
 H : HindIII  
 K : KpnI  
 M : SmaI  
 O : XhoI  
 P : PstI  
 S : SalI  
 T : Tth111I  
 V : PvuII  
 X : XbaI  
 II : BstEII

Ap : Ampicillin resistance gene  
 Km : Kanamycin resistance gene

P<sub>lac</sub> : Promoter of E.coli lac operon

kb : Kilo base pairs



**Figure 7:** Restriction map of pDM6. See figure 6 for legends to symbols.



European Patent  
Office

# EUROPEAN SEARCH REPORT

Application number

DOCUMENTS CONSIDERED TO BE RELEVANT			EP 89114632.6
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.) <sup>5</sup>
D, X	FR - A1 - 2 581 653 (ASAHI KASEI KOGYO KABUSHIKI KAISHA) * Abstract *	1, 11-13	C 12 N 15/60 C 07 H 21/04 C 12 N 1/20 C 12 N 9/88 C 12 P 13/04
D, X	EP - A1 - 0 143 195 (AJINOMOTO CO., INC.) * Claims *	1, 11-13	C 12 P 13/08 C 12 P 13/12 C 12 P 13/06 C 12 P 13/14
A	EP - A2 - 0 179 338 (AJINOMOTO CO., INC.) * Abstract; pages 18-20 *	1, 11-13	C 12 P 13/24 C 12 P 13/10 // (C 12 N 1/20 C 12 R 1:15) (C 12 N 1/20 C 12 R 1:13)
			TECHNICAL FIELDS SEARCHED (Int. Cl.) <sup>5</sup>
			C 12 N C 07 H C 12 P
The present search report has been drawn up for all claims			
Place of search VIENNA		Date of completion of the search 21-11-1989	Examiner WOLF
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

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